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ORIGINAL PAPER

Brewers' Spent Yeast and Grain Protein Hydrolysates as Second-Generation Feedstuff for Aquaculture Feed

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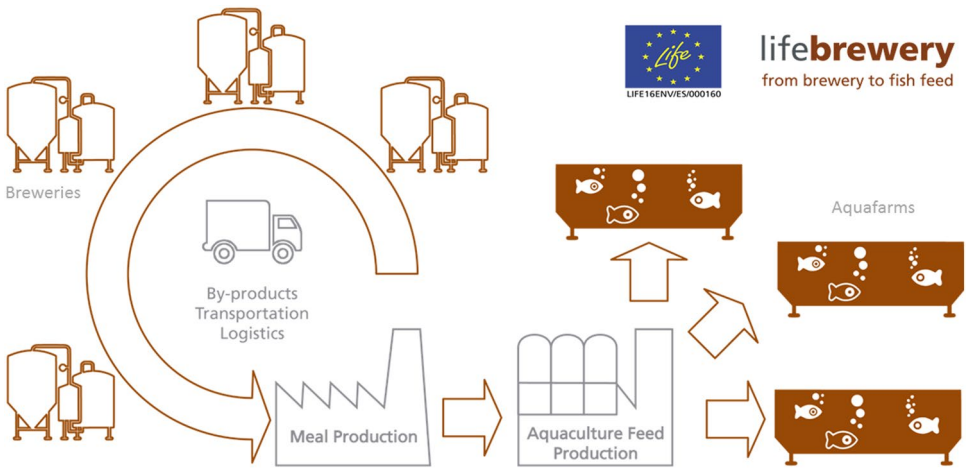
Abstract

Aquafeeds are formulated to contain all the essential nutrients that fishes need to keep healthy. They are highly dependent on marine ingredients: fish meal and oil. Hence, alternative ingredients which successfully replace these marine ingredients are required to result in sustainable and economical feeds. In this context, brewers' by-products arise as alternative potential ingredients to fish meal in aquafeed due their availability and nutritional content. However, reducing aquaculture's dependence on marine resources depends not only on developing alternative ingredients but also on improving their nutritional efficiency. In this context, Life Brewery project (LIFE16ENV/ES/000160) proposes an enzymatical hydrolysis step prior to the stabilization process to improve the digestibility of brewers' by-products and, therefore, increases the assimilation of nutrients by fishes. Hence, optimum hydrolysis conditions for both brewers' spent grain and yeast have been defined by comparing different enzymes combination and hydrolysis conditions at laboratory scale. Afterwards, selected enzymes and conditions have been validated at industrial scale. Finally, the digestibility of different experimental diets containing both hydrolysed and non-hydrolysed ingredients from brewers' waste has been determined with positive results. Obtained results showed that the inclusion of brewers' spent yeast and spent grain in aquafeeds for gilt-head sea bream show a good protein digestibility and can be considered as suitable ingredients to successfully replace fish meal in aquafeed diets.

Graphic Abstract



Keywords Brewers' by-products · Valorisation · Hydrolysis · Alternative ingredients · Sustainability · Circular economy

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Statement of Novelty

Brewers' by-products have lower intestinal absorption rate in fishes compared with marine ingredients. An innovative process for their use in aquafeed has been optimised and validated in gilt-head seabream. It includes hydrolysis and enhanced drying which improves the ingredient digestibility and the energy efficiency of their stabilization.

This is the first time that brewer spent grain is tested as an alternative ingredient to substitute marine ingredient (fish meal) in aquaculture diets, whereas brewer spent yeast have been studied but it stills not been industrialized.

This innovative process will allow to increase the inclusion rate of these alternative ingredients in aquafeed diets. Their availability in huge amounts will boost the sustainability of the aquaculture sector that is in an increasing tendency.

Introduction

Aquaculture sector continues to grow faster than other major food production sectors. The average annual growth rate during the period 2001–2016 was 5.8% [1]. In this context, aquafeeds are specially formulated to contain all the essential nutrients that farmed fishes need to keep healthy. They are highly dependent on marine ingredients: specially fish meal (FM) and fish oil (FO). According to IFFO, approximately 69% of FM and 75% of FO are utilized in aquaculture production [2]. However, the global fish meal production remains stable over the years or it does not grow at the same rate as demand, with small oscillations due to natural phenomenon such as El Niño phenomenon [1]. Consequently, an increased demand of marine origin ingredients coupled with the stagnation of the recent global fish meal production makes necessary to develop alternative ingredients to successfully replace these marine components with non-traditional sources.

Moreover, according to a recent study, replacing FM by other alternative ingredients, such as soybean or rapeseed, has enormous potential to reduce the environmental impact per tonne of aqua-feed in both Acidification potential (AP); Global Warming potential (GWP); Eutrophication potential (EP) or Land competition (LC) [3]. Thus, in case of the GWP, the fish meal standard trout feed has an impact of 1797 kg CO_2 equivalent per ton while the soybean meal and rapeseed meal based aquafeeds have 1019.65 and 1037.13 kg CO_2, respectively. Consequently, alternative ingredients are also required to result in sustainable feeds.

Within this framework, brewers' by-products arise high potential to be reuse as an alternative raw material for aquafeed due to their availability and their nutritional value. After China, the European Union (EU) is the 2nd largest beer producer in the world, ahead of USA, Brazil and Russia. According to Eurostat, over 40 billion litres of beer were produced in the European Union (EU) in 2018 [4]. The largest volume of solid by-products produced by breweries are brewers' spent grains (BSG) (80% of total solid by-products), followed by brewers' spent yeast (BSY) (10%). So, given EU beer production in 2018, about 7 million tons of BSG (14–20 kg per Hl of beer) and 0.9 million tons of BSY (2.0–4.0 kg per Hl of beer) were generated [5–8]. BSY and BSG are often conventionally reused as animal feed and, in some cases, bioethanol production or landfill refuge [9, 10]. This involves the loss of a valuable product. In addition, the use of these by-products as a direct supply for animal feed without any treatment depends on many factors which can significantly limit their feasibility and, in many cases, can make them even unsustainable. The high moisture content of these by-products leads to a rapid microbial spoilage. Thus, these by-products must be stabilized within the first 48 h to avoid the emergence of degrading microorganisms that would make them unsuitable for the valorisation [11, 12].

Regarding to the nutritional value of brewers' by-products, the chemical composition of BSG is characterized by a high-water content (75–80%) and a high protein content (18–35.4%, w/w). The amino acids content represents approximately 30% of its total protein content. Lysine accounts for 14.3% of the total protein content. Other amino acids in significant quantity are leucine, phenylalanine, isoleucine, threonine and tryptophan. Moreover, the presence of polysaccharides (e.g. β-glucans) and phenolic compounds have already demonstrated to have health benefits [7]. In the case of BSY, its chemical composition is characterized by a high-water content (85–90%) and the presence of carbohydrates, proteins, free amino acids, ash, vitamins, and fatty acids. The amino acids found in BSY are leucine, lysine, tyrosine, arginine, cysteine, histidine, isoleucine, methionine, phenylalanine, threonine, tryptophan and valine, which makes the BSY an excellent source of high-quality protein. In addition, BSY cell walls contains β-glucans (8%, w/w dry weight) and the external layer is formed by mannoproteins. These two compounds have immunomodulatory, antimutagenic and anticarcinogenic activities [7]. Therefore, the nutritional value of BSG and BSY arises high potential to be alternatives ingredient to reduce the high dependence on marine resources of aquafeeds. This reduction not only depends on developing alternative ingredients but also on improving their nutritional efficiency.

The animal origin proteins are nutritionally superior to plant origin ones because of the better proportion of essential amino acids and do not contain anti nutritionals [13, 14]. Moreover, the suitable level of substitution of FM and FO

by these new alternative ingredients depends on the target species [15, 16]. Therefore, increasing the digestibility of these alternative ingredients will increase the assimilation of nutrients by fishes and, thus, the viability of their inclusion on aquafeeds. In this sense, a previous hydrolysis step of the ingredients arises enormous potential to improve their physical and biochemical properties, which means a better intestinal absorption [17–19]. The hydrolysis of proteins involves the break of peptide bonds in proteins to obtain different molecular weights of peptides and free amino acids. This process must be always adapted to the characteristics of both the initial product to be hydrolysed and the final product to be obtained. In the case of the production of protein hydrolysates from animal by-products rich in keratin, chemical hydrolysis in acid medium is normally used. In the other cases, such as other type of animal by-products or vegetable by-products (e.g. soybeans, cereals, yeast) enzymatic hydrolysis is widely used [20].

Finally, the obtained hydrolysates are characterized by their high moisture content which makes them rapidly biodegradable due to the microbial activity [11, 12]. Thus, their stabilization through a drying process is of utmost importance. However, traditional drying processes (rotary drum, fluidized bed, etc.) are energy intensive processes and, consequently, most of them economically unfeasible at industrial level. Hence, a low energy consumption drying process is necessary to guaranty the profitability of the development of brewers' by-products-based ingredients.

This study is focused on assessing the BSG and BY as alternative ingredients in aquafeeds formulations to replace the marine origin components. Specifically, the objectives are to determine the optimum hydrolysis process by comparing different enzymes combinations and hydrolysis conditions, to develop an efficient and sustainable drying process and to assess the increased digestibility of hydrolysed ingredients comparing to non-hydrolysed through feed efficiency growth trials with fishes.

Material and Methods

Analytical Methods

Characterization of Hydrolysates

The total protein content in the hydrolysis kinetic studio was performed by Kjeldahl Method. The analytical method for determining the molecular profile distribution of final hydrolysates was Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Thus, in dry and semi-dry samples, equal amounts of 0.5 g of each sample were collected. After collection, a soft extraction was performed using ACN: H_2O [1:1] with 0.1% TFA. Extracted samples were loaded in SDS-PAGE (12% acrylamide, 1 mm width, 10 lanes) for 1.5 h at 125 V. In the case of liquid samples, 100 µl were collected, and loaded in each corresponding SDS-PAGE. After running, samples gels were fixed and stained with Coomassie blue stain overnight, and finally, destained and stored with water at 4 °C. All process was performed following the Laemmli standardized methodology [21].

Characterization of Final Ingredients, Experimental Feeds & Faeces for Digestibility Studio

The analytical methods for the characterization of the final ingredients of brewers' by-products-based meals, the experimental feeds used in the digestibility study and the faeces obtained in the digestibility trial were selected following the official methods of analysis published by AOAC (2000) [22]: Dry matter (%); Crude protein (%); Crude fat and Ether extract (%); Ash (%); Gross energy (cal/g); Crude fibre (%); Carbohydrates and Starch (%); Phosphorus (%); Vitamin B2 (ppm). In addition, the Beta-glucan (g/100 g DM) content was determined by enzymatic commercial kit [23]; the total amino acids content (%) was analysed by pre-derivatization with o-phthalaldehydet3-mercapto propionic acid (OPA/MPA) and 9-fluorenyl-methylchloroformate (FMOC) and HPLC separation by DAD/FL detection [24] and, finally, the Yttrium was analysed by inductively coupled plasma mass spectrometry [25].

Hydrolysis Kinetic and Effectiveness of Proteolytic Activity

The hydrolysis kinetic studio was performed at laboratory-scale stirred batch reactor, Symphony 7100 Bathless Dissolution Distek® equipment, with a total working capacity volume of 1 L. The hydrolysis conditions (enzyme dosage, temperature and pH) were established based on the enzyme product data sheets provided by Novozymes. The ratio enzyme/product dry matter was 1%, temperature 55 °C and pH 6. For BSG hydrolysis kinetic studio, 400 g of BSG were mixed with 400 ml of water (solid/liquid ratio 1/1). For BSY, it was not necessary to add water since it could be easily stirred.

The selection of the commercial enzymes for BY hydrolysis was based on the hydrolysis objectives: on the one hand, to hydrolyse the protein with the aim of increasing the protein digestibility of ingredients in fishes [17–19] and, on the other hand, to increase the palatability. In this sense, there are some studies that shown the importance of reducing bitterness in the acceptance of new diets by fishes [17, 26, 27]. Thus, the selected commercial enzymes were: Protamex® and Flavourzyme® provided by Novozymes company.

Within this framework, three different enzymatic treatments were studied at laboratory scale:

- *Treatment 1* Protamex® and Flavourzyme® enzymes simultaneous addition
  The objective of this treatment was to assess the protease activity of both enzymes at the same time, taking advantage of the improvement of the reduction of bitterness with the Flavourzyme® enzyme.
- *Treatment 2* only Protamex® enzyme addition
  The objective of this treatment was to assess the protease activity of the Protamex®, without the action of Flavourzyme® for reducing the bitterness of final product.
- *Treatment 3* only Flavourzyme® enzyme addition
  The objective of this treatment was to assess if the protease activity of Flavourzyme® was high enough compared with the treatment 2 and treatment 3.

The selection of the commercial enzymes for BSG was based on the hydrolysis objectives that, in case of BSG, was not only to hydrolyse the protein but also the fibre. There are some studies that shown that fibre is a potential antinutritional for fishes [14, 28]. Thus, the selected commercial enzymes were: Celluclast ® and Protamex® provided by Novozymes. Within this framework, three different treatments were studied at laboratory scale:

- *Treatment 1* Celluclast® and Protamex® enzymes sequential addition.
  The objective of this treatment was to test the increase of the access to protein of Protamex® enzyme after the fibre hydrolysis by Celluclast®.
- *Treatment 2* Celluclast® and Protamex® enzymes simultaneous addition.
  The objective was to compare the effectiveness regarding treatment 1.
- *Treatment 3* only Protamex® enzyme addition
  The objective was to compare the effectiveness of Protamex® enzyme without previous fibre hydrolysis.

The hydrolysis kinetic of each treatment was studied by analysing the total protein content at different hydrolysis times by Kjeldahl method. The experimental concentration of protein released (PR) from the different enzymatic treatments were smoothed by using the biologistic (the sum of two logistic models) equation [29]:

$$[PR] = \frac{K_1}{1 + c^{(c1-b1t)}} + \frac{K_2}{1 + c^{(c2-b2t)}}$$

where K_1 and K_2 are, respectively, the maximum concentrations of protein released (%) in the first and second enzyme kinetics phase, b_1 (h^{-1}), b_2 (h^{-1}), c_1 (dimensionless) and c_2 (dimensionless) are logistic parameters and t is the time (h). When the kinetics of protein released displayed only one enzyme kinetics phase, the bi-logistic models takes form of a simple logistic model ($K_2 = 0$).

The effectiveness of each hydrolysis treatment was assessed by determining the molecular profile distribution of different hydrolysates by SDS-PAGE. With the aim of simulating the production conditions of the final ingredients described in "Scaling-Up of Ingredients Production" section, the hydrolysates from each hydrolysis treatment were mechanical dewatered to assess the amount of soluble protein that is lost with the liquid fraction.

Scaling-Up of Ingredients Production

The hydrolysis process was scaled-up in 1 m^3 stirred reactor for both by-products. The hydrolysis conditions were the same used at the laboratory. The drying process consisted of a first mechanical dewatering to reduce the humidity below 60%, which involves a low energy demand and, therefore, a reduction of the energy necessary for thermal drying in the second step. The second phase applies a thermal drying to reduce moisture content under 10%. From both fractions resulting from the centrifugation, the semi-solid one was used for aquafeeds formulation.

Mechanical dewatering technologies selection depends on the physical properties of the targeted products. In case of BSG, the selected technology was a filter centrifuge whereas in case of BY the selected technology was a decanter centrifuge:

- Filter centrifuge is based on the principle of gravity and centrifugal force. It puts an object in rotation around a fixed axis and applies a force perpendicular to the axis of spin (outward). The centrifugal force makes denser particles to move outward in the radial direction and less dense particles to the centre. Therefore, in case of BSG, the solid particles are trapped in a separation mesh that can have different pore size due to the centrifugal force and the liquid flows out from the upper side of the separator.
- Decanter centrifuge is based on the principle of separation via buoyancy to separate continuously solid materials from liquids. Considering that a denser particle falls to the bottom, while a less dense particle is suspended above it, a decanter centrifuge increases the rate of settling using continuous rotation to reduce the settling time of the particles.

Thermal drying technology selection depends fundamentally on its efficiency. Thus, flash drying technology was selected

as the most appropriate thermal drying technology for drying both BSY and BSG.

- Flash dryer is based on the instant, self-regulating and continuous drying of wet solids. It is a high thermal efficiency technology which combines the effect of turbulence with the high-speed movement of wet solid particles to dry them instantly. The products are broken in the drying chamber (Fig. 1) and the surface area of particles increases significantly, and therefore, decreasing the required energy to dry them. Finally, a minimum heating during a short time of residence—fractions of a second—makes it suitable for temperature-sensitive products maintaining the nutritional value and food security [30].

The equipment used for the scaling up of the ingredients production were:

- Decanter centrifuge "GEA CA 225–00-33", owned by GEA Westfalia (Barcelona, Spain).
- Filter centrifuge "RINA 200F 1000 S PI", owned by Riera Nadeu company (Granollers, Spain).

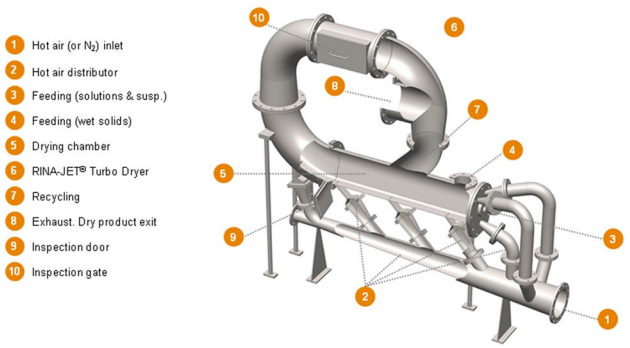

Fig. 1 Structure of the RINA-JET flash dryer technology

- Flash dryer "RINA-JET S-1008", owned by the Riera Nadeu company (Granollers; Spain).

Determination of Protein Digestibility in Fishes

The selected fish species was gilt-head sea bream (*Sparus aurata*) as a model of Mediterranean aquaculture.

Experimental Diets Determination

A commercial-based diet for gilt-head sea bream using fish meal (Corpesca Super Prime LT, Chile) was formulated and extruded at IRTA facilities using an extruder (Mabrik; MDG 40 model). To this mixture 20 g/kg or Yttrium oxide (Sigma, Spain) was added as an inert marker for the evaluation of the apparent digestibility coefficient (ADC).

In addition, four experimental diets were also produced by mixing 700 g/kg of the basal mixture and 300 g/kg of each test ingredient (BSY and BSG, hydrolysed and unhydrolyzed). The reference diet contained 209 g/kg of starch to enable extrusion of the pellets which was hindered due to its high fibre content.

All the diets were formulated to be iso-protein and iso-lipidic and are presented in Tables 1 and 2. The inclusion of BSY and BSG resulted in experimental diets that had 393 to 420 g/Kg crude protein, 218 to 224 g/Kg crude fat, 17 to 19 MJ/Kg gross energy, reflecting the similarity among the diets.

Digestibility Trials with Fishes

The protein digestibility trials of obtained ingredients with fishes were carried out at IRTA facilities using recirculation (RAS) systems. The fishes were purchased in a commercial farm (Piscimar, Castellón, Spain), transported by road to IRTA facilities, acclimatized for 15 days and then randomly distributed in fifteen 500 L tanks with individual faeces sedimentation columns in the outflow of the tanks. Twenty-five

Table 1 Formula of the experimental diets for protein digestibility determination in gilt-head sea bream

Ingredients	Control	Spent yeast 30%	Hydrolysed Spent yeast 30%	Spent grain 20%	Hydrolysed Spent grain 20%
Fish meal 70 LT	60.00	40.00	42.00	50.00	50.00
Wheat starch	20.95	9.45	7.45	10.00	10.00
Spent yeast	–	30.00	–	–	–
Hydrolyzed spent yeast	–	–	30.00	–	–
Spent grain	–	–	–	20.00	–
Hydrolyzed spent grain	–	–	–	–	20.00
Fish oil	18.00	19.50	19.50	16.50	17.00
Vit and Min Premix PV01	1.05	1.05	1.05	1.05	1.05
Yttrium	0.02	0.02	0.02	0.02	0.02

Table 2 Composition of the experimental diets for protein digestibility determination in gilt-head sea bream

Parameters	Control	Yeast 30%	Hydrolysed yeast 30%	Spent grain 20%	Hydrolysed spent grain 20%
Dry matter (DM, g/Kg)	978.30 ± 3.09	979.20 ± 2.36	976.60 ± 5.46	980.50 ± 5.50	978.10 ± 8.50
Ash (g/Kg DM)	98.80 ± 0.98	83.20 ± 0.77	78.70 ± 0.76	93.60 ± 4.24	100.60 ± 1.07
Crude protein (g/Kg DM)	419.80 ± 3.39	413.30 ± 0.16	418.20 ± 2.49	417.70 ± 3.51	392.80 ± 0.70
Crude fat (g/Kg DM)	218.42 ± 3.29	223.94 ± 1.45	234.04 ± 5.71	219.83 ± 2.04	221.40 ± 1.59
Carbohydrates (g/Kg DM)	215.10 ± 8.42	218.50 ± 16.36	197.00 ± 4.22	130.20 ± 9.07	166.20 ± 11.46
Gross energy (MJ/Kg DM)	18.65 ± 0.08	18.89 ± 0.19	19.04 ± 0.32	17.25 ± 0.22	17.53 ± 0.23

fishes with a body weight of 253.01 ± 27.68 g were randomly distributed in the tanks connected to the RAS systems and kept at 20 °C under natural light.

The experimental diets were randomly assigned to the tanks and fed in triplicate. Fish were fed 100 g of the feeds once daily for 2 weeks before faecal collection. Gilt-head sea bream faeces were collected in the sedimentation columns for 3 alternate days. Gilt-head sea bream were also fed once per day during the collection period and the tanks cleaned to avoid any uneaten feed in the tanks and in the faecal collectors. Faecal samples were stored at − 20 °C until chemical analyses.

The apparent digestibility coefficients (ADC) of the experimental diets were calculated according to Maynard et al. methodology [31].

$$\text{ADC}\,(\%) = 100 \times \left(1 - \left(\text{dietary } Y_2O_3 \text{level/faeces } Y_2O_3 \text{level}\right)\right) \times \left(\text{faeces nutrient or energy level/dietary nutrient or energy level}\right)$$

The ADC of the test ingredients were estimated according to National Research Council method [32]

$$\text{ADC}_{\text{BSG}}(\%) = \text{ADC}_{\text{test}} + \left[\left(\text{ADC}_{\text{test}} - \text{ADC}_{\text{ref}}\right) \times \left(\left(0.8 \times \text{D}_{\text{ref}}\right) / \left(0.2 \times \text{D}_{\text{ing}}\right)\right)\right]$$

$$\text{ADC}_{\text{BSY}}(\%) = \text{ADC}_{\text{test}} + \left[\left(\text{ADC}_{\text{test}} - \text{ADC}_{\text{ref}}\right) \times \left(\left(0.7 \times \text{D}_{\text{ref}}\right) / \left(0.3 \times \text{D}_{\text{ing}}\right)\right)\right]$$

where ADC_{test} = ADC (%) of the experimental diet; ADC_{ref} = ADC (%) of the reference diet; D_{ref} = g/Kg nutrient (or MJ/Kg gross energy) of the reference diet (DM basis); D_{ing} = g/Kg of nutrient (or MJ/Kg gross energy) of the test diet (DM basis)

Results and Discussion

Hydrolysis Kinetics

With regards to mathematical model fitting, Table 3 shows the results of each parameters for BSY and BSG hydrolysis and the R^2 values between experimental and predicted values.

As seen in the table above, the experimental data fitted very well to the model with R^2 values > 0.90. For BSY, this fitting was slightly higher comparing to BSG.

The hydrolysis kinetic studio results show how protein is partially solubilized due to enzymes action. Figure 2 shows the protein solubilization (decrement of protein in the solid phase) from both BSY and BSG for the different treatments:

Table 3 Kinetic parameters and the R^2 values for BSY and BSG hydrolysis

Substrate	Treatment	K_1 (%)	K_2 (%)	c_1	c_2	b_1 (h^{-1})	b_2 (h^{-1})	R^2
BSY	Treatment 1	5.63	0.995	0.000	1.370	0.234	0.000	0.995
	Treatment 2	6.84	0.000	0.472	0.142	0.233	0.000	0.998
	Treatment 3	7.81	0.000	0.779	1.481	0.153	0.000	0.977
BSG	Treatment 1	0.12	1.534	7.956	0.598	3.766	0.091	0.993
	Treatment 2	0.11	1.231	5.321	0.456	2.393	0.086	0.993
	Treatment 3	3.01	12.815	53.936	2.660	2.442	0.023	0.945

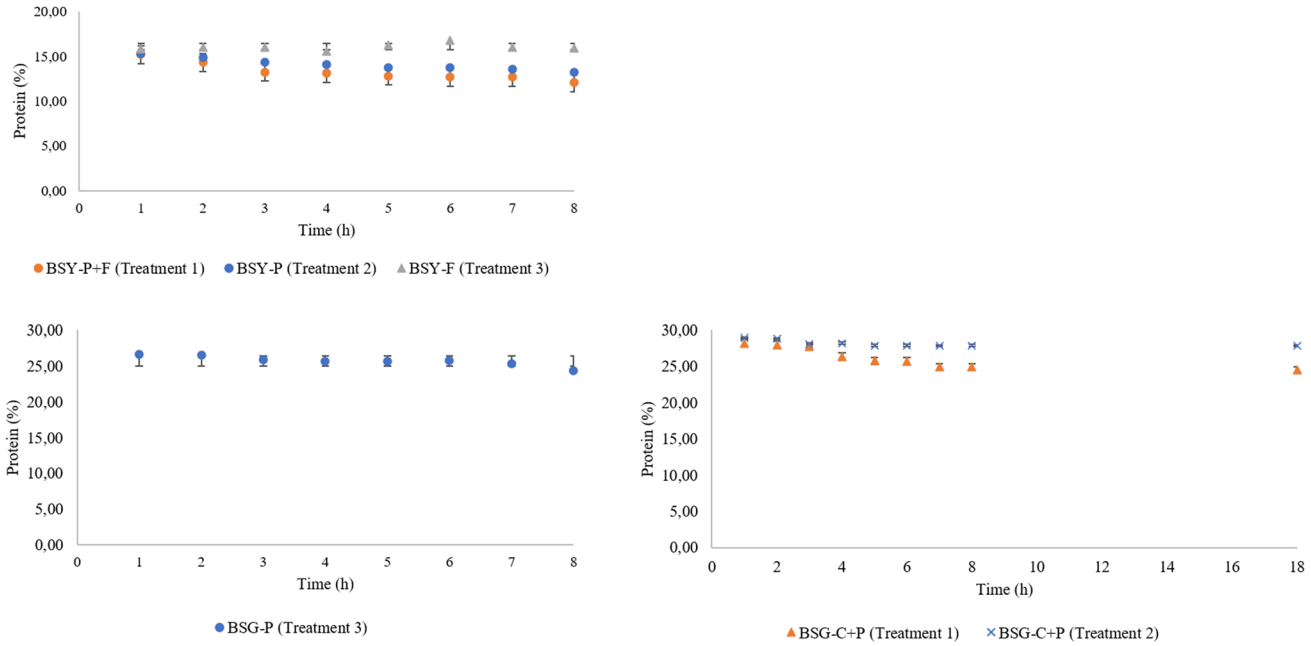

Fig. 2 Protein solubilization in the solid phase of brewer's spent yeast (BSY) and spent grain (BSG)

When Celluclast® is added longer periods of hydrolysis (at least 12 h) are required to solubilize the fibre. From the graphics above, it can be concluded that only Protamex® addition was not enough to perform the protein solubilization. For both BSY and BSG, the addition of a second enzyme (Flavourzyme® for BSY or Celluclast® for BSG) increased slightly the protein solubilization rates. Despite the solubilization of protein is relatively low (up to 20% and for BSY and 18% for BSG), it was demonstrated that nutritional efficiency can be increased considerably depending on the size of the final hydrolysis products. Thus, it is utmost of importance to assess the hydrolysis effectiveness by determining the different molecular sizes of these hydrolysis products.

Effectiveness of Proteolytic Activity

Spent Yeast

The results of effectiveness of each hydrolysis treatment by SDS-PAGE (Fig. 3) showed that, in H3.2 lane (semi-solid fraction), protease activity was low, with dense high molecular mass bands (above 25 kDa). On the other hand, H1.2 and H2.2 (semi-solid fractions) look very similar, with apparently higher protease activity, in contrast to H3.2 (semi-solid fraction). In all cases, liquid fractions (H1.1, H2.1 and H3.1 lanes) showed very little amount of protein.

Considering all the above results, the treatment 1 (Protamex® and Flavourzyme® enzymes simultaneous addition) has been selected as the most appropriate for the protein hydrolysis of BSY to produce a new ingredient for aquaculture feed. The treatment 3 (only Flavourzyme® enzyme addition) has been rejected due to the low protease activity. In addition, although the effectiveness of the treatment 2 (only Protamex® enzymes addition) is quite similar to treatment 1, and therefore, the expected protein digestibility improvement in fishes should be similar, the expected reduction in the bitterness related to treatment 1 due to the activity of Flavourzyme® enzymes has been considered of high importance to ensure the viability of this new ingredient for aquaculture feed application.

Spent Grain

The results of effectiveness of each hydrolysis treatment by SDS-PAGE (Fig. 4) showed that the protease activity of samples corresponding to Control, Treatment 1, Treatment 2 and Treatment 3 were very similar, with a similar pattern in each SDS-PAGE lane. However, some slight differences can be observed in terms of intensities at the band of 50 kDa. The H1.2 lane of the hydrolysis treatment 1 presents lesser intensity, and therefore, more hydrolysis degree comparing to the rest of the lanes.

Considering the obtained results, treatment 1 (Celluclast® and Protamex® enzymes sequential addition), treatment 2 (Celluclast® and Protamex® enzymes simultaneous addition) and treatment 3 (only Protamex® enzyme addition) are viable for the protein hydrolysis of BSG to produce a new ingredient for aquaculture feed.

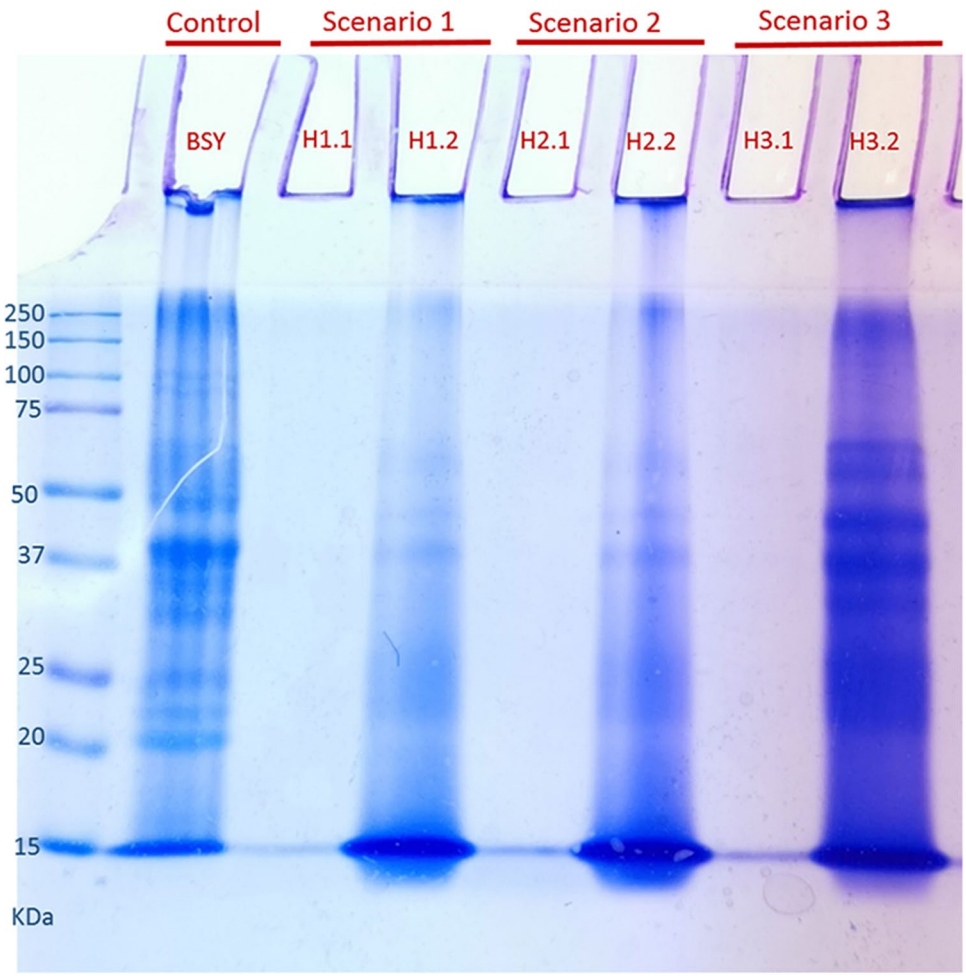

Fig. 3 Molecular profile distribution of different brewer's spent yeast hydrolysis treatments by sodium dodecyl sulphate polyacrylamide gel electrophoresis method

However, provided fibre is considered an anti-nutritional parameter for fishes [14, 28], the fibre hydrolysis related to the activity of Celluclast® enzyme has been considered of high importance to ensure the viability of this new ingredient for aquaculture feed application. Thus, the treatment 3 has been rejected for this proposal.

Within this context, the treatment 1 (Celluclast ® and Protamex® enzymes sequential addition) was selected as the most appropriate for the hydrolysis of BSG to produce a new ingredient for aquaculture feed due to the light tendency to a higher proteolytic activity in the band of 50–150 kDa.

Scaling-Up of Ingredients Production

Spent Yeast

The scaling up of 2 different ingredients from BSY: hydrolysed (treatment 1) and non-hydrolysed consisted of a first mechanical dehydration to reduce as much as possible the humidity (less than 60%) and a second thermal drying to reduce moisture content below 10%. Obtained ingredients have been used in the determination of protein digestibility in fishes in Sect. 3.3.1. The characterization results of these ingredient are shown in the Table 4.

The Fig. 5, related to the SDS-PAGE analysis of the protein hydrolysis effectiveness of the results at laboratory scale [H1.1 (liquid fraction) and H1.2 (semi-solid fraction)] comparing with the industrial scale ones [H1.3 (liquid fraction), H1.4 (semi-solid fraction) and H1.5 (solid fraction)], showed very similar SDS-PAGE patterns, which means similar levels of protease activity. The amount of hydrolysis seems the same, with similar composition of remaining high molecular mass bands. This shows that the scaling up of the treatment 1 at semi-industrial scale was performed correctly.

In addition, the stabilization process of the semi-solid fraction (H1.4) to obtain the dried ingredient (H1.5) showed no significant differences. Thus, the stabilization process does not affect to the protein content and its molecular profile distribution. Finally, as seen in Fig. 3, liquid fractions (H1.1 and H1.3) showed very little amounts of protein. This involves that the stabilization process proposed for drying BSY in an efficient way is compatible with the hydrolysis process since the loss of soluble protein in the liquid fraction is minimum.

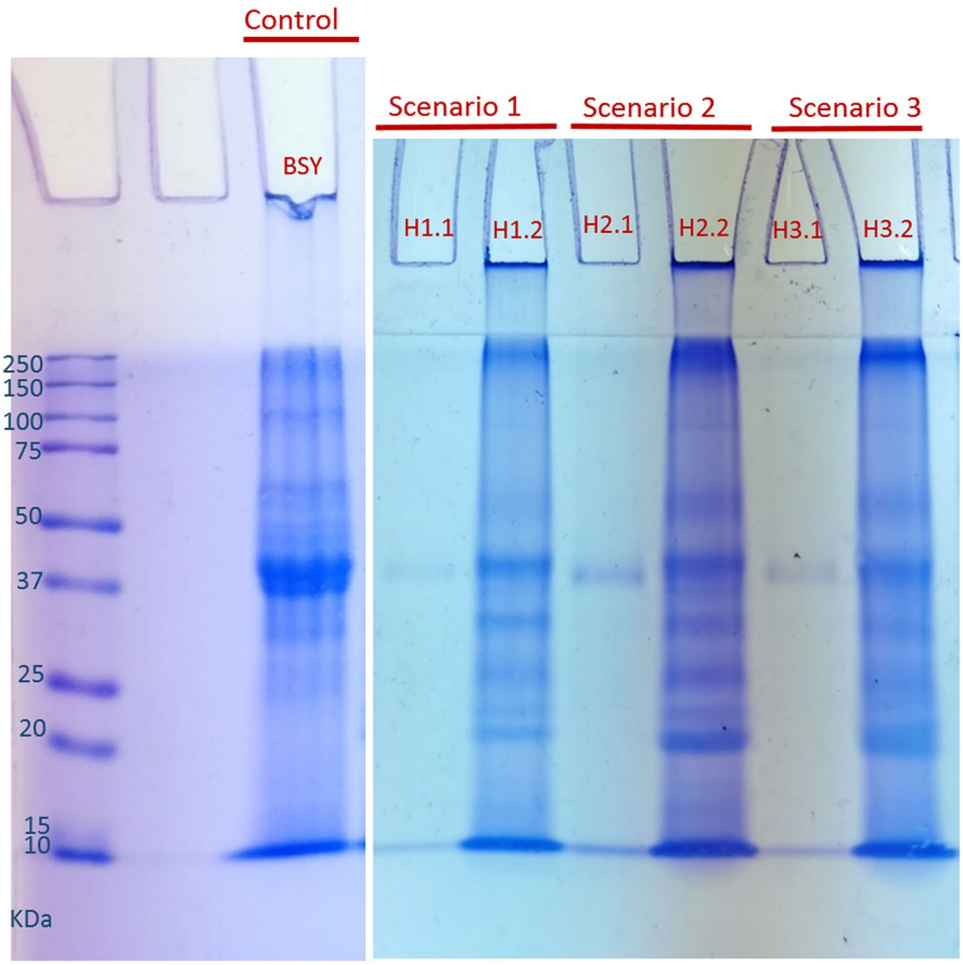

Fig. 4 Molecular profile distribution of different BSG hydrolysis treatments by polyacrylamide gel electrophoresis method

Spent Grain

The scaling up of 2 different ingredients from BSG: hydrolysed (treatment 1) and non-hydrolysed consisted of a first mechanical dehydration to reduce as much as possible the humidity (less than 60%) and a second thermal drying to reduce moisture content below 10%. Obtained ingredients have been used in the determination of protein digestibility in fishes in Sect. 3.3.2. The characterization results of these ingredient are shown in the Table 5

The Fig. 6, related to the SDS-PAGE analysis of the protein hydrolysis effectiveness of the results at laboratory scale [H1.1 (liquid fraction) and H1.2 (semi-solid fraction)] comparing with the industrial scale ones [H1.3 (liquid fraction), H1.4 (semi-solid fraction) and H1.5 (dried fraction)] shown that a clear hydrolysis has been performed, in comparison to control (BSG). But, at industrial scale (H1.3 to H1.5), the hydrolysis seems to be higher than at laboratory scale (H1.1 and H1.2). H1.2 shows higher intensity bands above 25 kDa than those in H1.4 and H1.5. This is clearly shown in corresponding densitograms. This shows that the scaling up of the treatment 1 at semi-industrial scale was performed correctly.

In addition, the stabilization process of the semi-solid fraction (H1.4) obtained by performing the hydrolysis treatment 1 to obtain the dried ingredient (H1.5) did not show differences. Thus, the stabilization process does not affect to the protein content and its molecular profile distribution.

Finally, as in Fig. 4, liquid fractions (H1.1 and H1.3) show very little amount of protein. This involves that the stabilization process proposed for drying BSG in an efficient way is compatible with the hydrolysis process since the loss of soluble protein in the liquid fraction is minimum.

Determination of Protein Digestibility in Fishes

Spent Yeast and Spent Grain

No mortality was observed during the trial. The apparent digestibility coefficients (ADC) of the experimental diets formulated with hydrolysed and non-hydrolysed BSY and BSG at inclusion level of 30% and 20% for gilt-head sea

Table 4 Brewers' spent yeast-based ingredients nutritional value

Parameter	Non-hydrolysed spent yeast	Hydrolysed spent yeast
Dry matter (%)	94.19	89.05
Crude protein (%)	45.07	41.24
Ether extract (%)	0.35	0.45
Ash (%)	3.99	3.87
Gros energy (cal/g)	4477	4238
Crude fibre (%)	0.64	0.62
Starch (%)	20.59	20.05
Phosphorus (%)	0.91	0.87
Vitamin B2 (ppm)	2.60	5.00
Total a.a. content (%)	40.60	36.63
Aspartic acid	4.51	4.08
Glutamic acid	5.68	5.19
Serine	2.39	2.16
Histidine	1.14	1.02
Glycine	1.78	1.63
Threonine	2.29	2.09
Arginine	2.37	2.12
Alanine	2.77	2.52
Tyrosine	1.62	1.40
Valine	2.50	2.25
Methionine	0.77	0.69
Phenylalanine	2.17	2.00
Isoleucine	2.21	2.02
Leucine	3.31	3.00
Lysine	2.99	2.57
Hydroxyproline	< 0.03	< 0.03
Proline	2.10	1.89

bream are presented in Table 6. This parameter reflects the capability of a certain species of fish to utilize the nutrients of an ingredient, which predicts its potential as a feedstuff.

Table 6 shows that in all the tested diets, the digestibility of protein was relatively high (71–85%) comparing to control diet. In this case, the results show acceptable digestibility for gilt-head sea bream indicating that BSY based ingredients (protein but also lipids—not shown-) are suitable for aquaculture nutrition. As expected, the diets which included the hydrolysed ingredients showed a growing trend in the digestibility comparing to non-hydrolysed. However, the one-way ANOVA with post-hoc Tukey tests (95% confidence level) demonstrated that there were not statistically significant differences in the ADC parameter between hydrolysed and non-hydrolysed diets for the same inclusion levels. This fact might be because of the final amounts of both hydrolysed and non-hydrolysed ingredients formulated in the diets are not high enough to appreciate the beneficial effects of the hydrolyzation over the digestibility. Thus, further studies would be required to analyse the digestibility of each ingredient.

According to these results, it can be concluded that the inclusion of BSY and BSG for growing gilt-head sea breams is suitable and, consequently both brewers' by-products can be used to replace some of the marine origin ingredients to produce more environmentally friendly diets. Some previous studies carried out with BSY with Mediterranean species (e.g. sea bass) [18, 33] showed also good digestibility results for non-hydrolysed and hydrolysed BSY. In this study, the use of BSY hydrolysates > 3000 KDa gave a value of 87.9 for ADC parameter [18].

Conclusions

The first conclusion from this study is that the production of alternative ingredients for aquaculture feed based on brewers´ by-products is feasible from the technical point of view. The stabilization process consisted of a first mechanical dehydration step to reduce the humidity below 60%, and secondly, a thermal drying process to reduce moisture content below 10%. This process has been demonstrated as appropriate for brewers based aquafeed ingredients production. The mechanical dewatering process involves less energy demand and, therefore, a reduction of the energy consumption which makes the whole process more sustainable since economic and environmental points of view.

Moreover, brewers´ by-products stand as potential alternative ingredients for replacing fish meal in aquaculture feed due to their wide availability across the European countries (over 40 billion litres of beer were produced in the European Union (EU) in 2018 [4]). Their high nutritional values (rich in vegetable origin protein) in conjunction with the positive digestibility results at pilot scale achieved in this study with gilt-head sea bream (*Sparus aurata*) makes these ingredients a real alternative to marine origin-based diets. However, further studies are required to assess the potential benefits of hydrolysed ingredients over the non-hydrolysed ones.

The proposed solution involves an increase of the economic and environmental sustainability of aquaculture sector by providing two new more economic and environmentally friendly protein sources that could replace fish meal in the near future. The reduction of aquaculture production costs will contribute to achieve the objectives established by the new European Common Fisheries Policy. Finally, the replacement of marine origin ingredients (fishmeal) will also significantly reduce the wild catches, which will contribute to achieve the goals defined in the Marine Strategy Framework Directive.

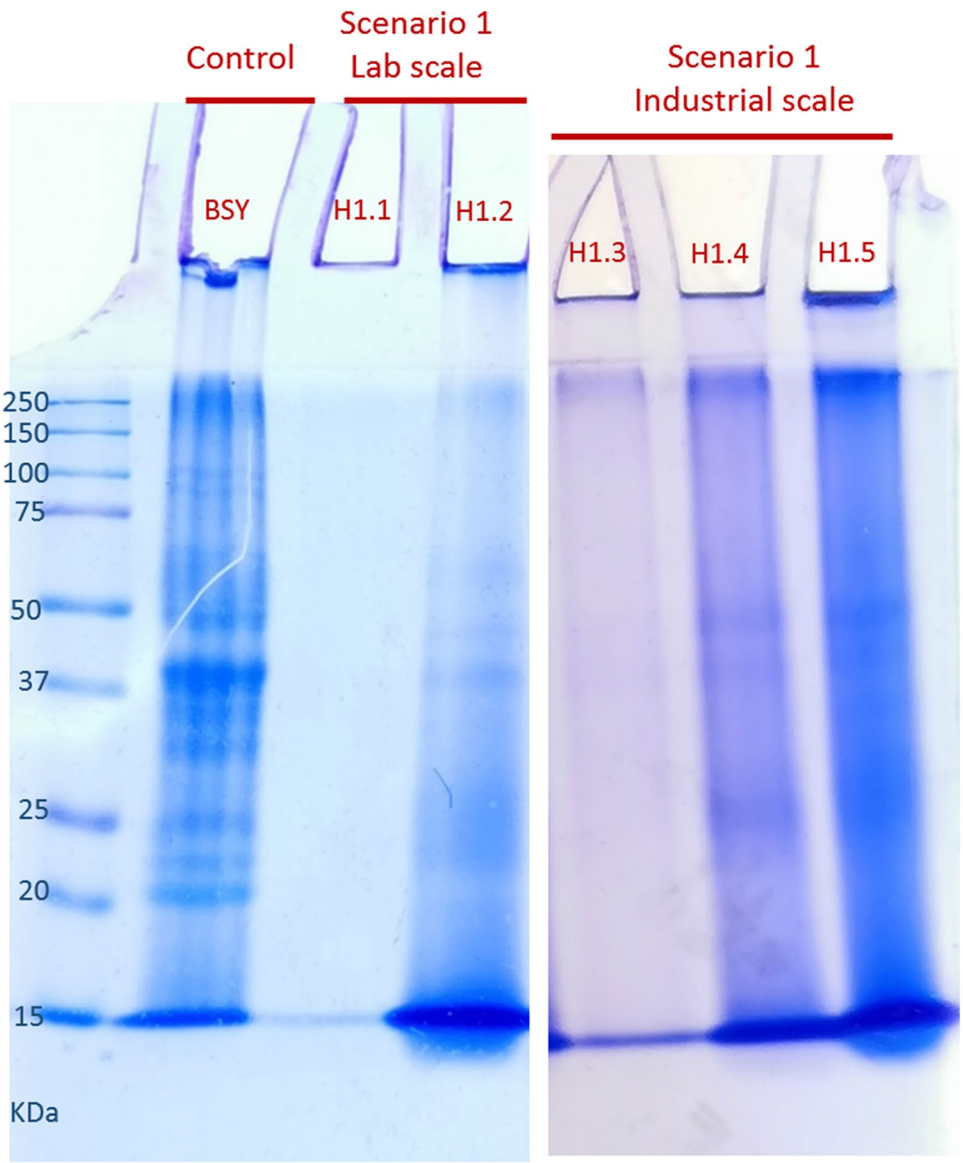

Fig. 5 Molecular profile distribution of the BSY hydrolysis treatment 1 at lab and industrial scale by sodium dodecyl sulphate polyacrylamide gel electrophoresis method

Table 5 Brewers' spent grain-based ingredients nutritional value

Parameters	Non-hydrolysed spent grain	Hydrolysed spent grain
Dry matter (%)	92.00	98.11
Crude protein (%)	22.73	21.38
Ether extract (%)	7.75	11.46
Ash (%)	3.60	5.99
Gros energy (cal/g)	4766	4838
Crude fibre (%)	17.28	16.28
Starch (%)	3.59	3.43
Phosphorus (%)	0.49	0.34
Vitamin B2 (ppm)	0.40	1.40
Total a.a. content (%)	22.51	18.67
Aspartic acid	1.58	1.42
Glutamic acid	4.82	3.62
Serine	1.03	0.86
Histidine	0.60	0.55
Glycine	0.81	0.78
Threonine	0.84	0.79
Arginine	1.18	1.05
Alanine	1.39	1.15
Tyrosine	0.88	0.79
Valine	1.17	1.03
Methionine	0.47	0.38
Phenylalanine	1.33	1.11
Isoleucine	0.94	0.79
Leucine	2.28	1.81
Lysine	0.88	0.72
Hydroxyproline	<0.03	0.03
Proline	2.31	1.79

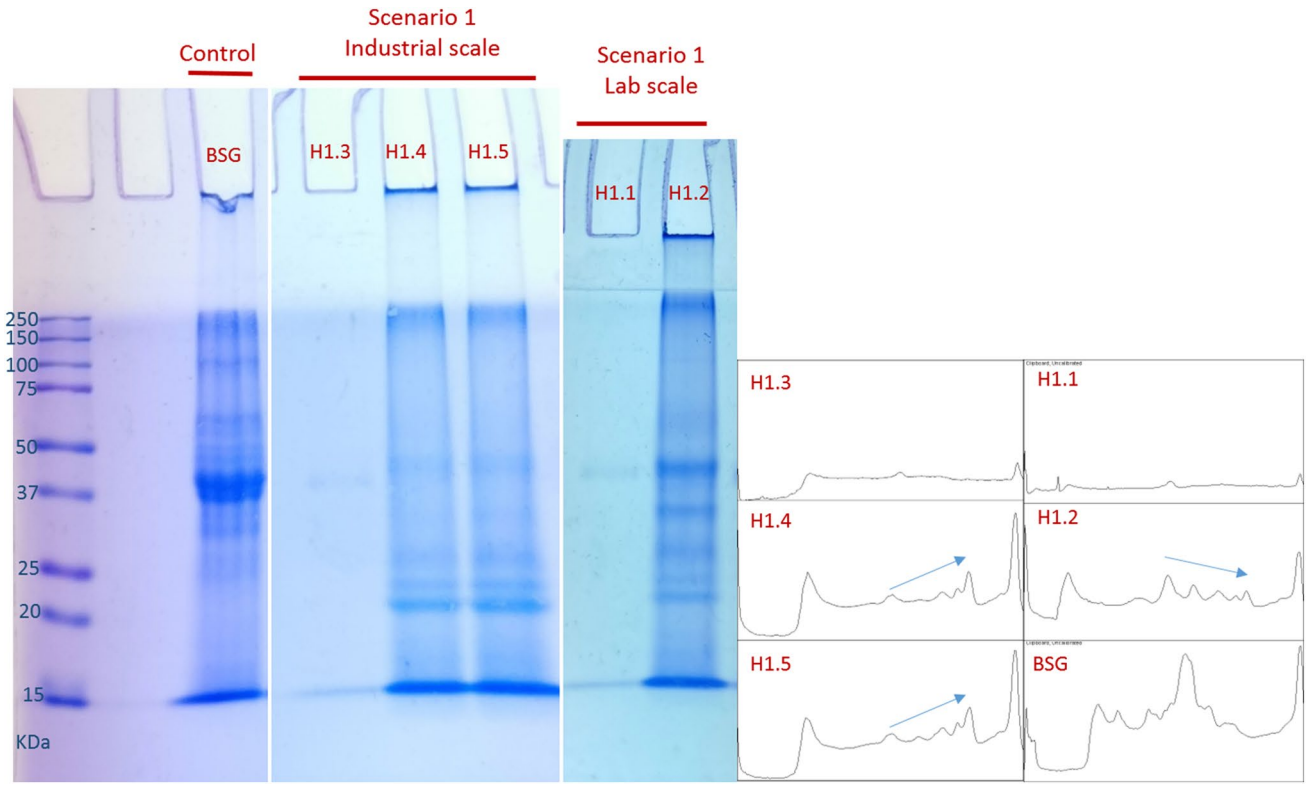

Fig. 6 Molecular profile distribution of the BSG hydrolysis treatment 1 at lab and industrial scale by SDS-PAGE method

Table 6 Apparent Digestibility Coefficients (ACD) of hydrolysed and non-hydrolysed BSY and BSG based experimental diets in gilt-head sea bream

Diet	Protein in faeces	Protein in diet	ADC	SD
Control	198.1 ± 0.40	419.80 ± 3.39	90.26[a]	0.11
Non-hydrolysed BSY 30%	262.4 ± 1.59	413.30 ± 1.16	71.76[b]	2.73
Hydrolysed BSY 30%	223.1 ± 2.79	418.20 ± 2.49	75.01[b]	1.27
Non-hydrolysed BSG 20%	118.2 ± 3.41	417.70 ± 3.51	84.01[c]	0.54
Hydrolysed BSG 20%	87.8 ± 0.90	392.80 ± 0.70	85.22[c]	0.31

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